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The *Saccharomyces cerevisiae ICL2* Gene Encodes a Mitochondrial 2-Methylisocitrate Lyase Involved in Propionyl-Coenzyme A Metabolism

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The *Saccharomyces cerevisiae ICL1* gene encodes isocitrate lyase, an essential enzyme for growth on ethanol and acetate. Previous studies have demonstrated that the highly homologous *ICL2* gene (YPR006c) is transcribed during the growth of wild-type cells on ethanol. However, even when multiple copies are introduced, *ICL2* cannot complement the growth defect of *icl1* null mutants. It has therefore been suggested that *ICL2* encodes a nonsense mRNA or nonfunctional protein. In the methylcitrate cycle of propionyl-coenzyme A metabolism, 2-methylisocitrate is converted to succinate and pyruvate, a reaction similar to that catalyzed by isocitrate lyase. To investigate whether *ICL2* encodes a specific 2-methylisocitrate lyase, isocitrate lyase and 2-methylisocitrate lyase activities were assayed in cell extracts of wild-type *S. cerevisiae* and of isogenic *icl1*, *icl2*, and *icl1 icl2* null mutants. Isocitrate lyase activity was absent in *icl1* and *icl1 icl2* null mutants, whereas in contrast, 2-methylisocitrate lyase activity was detected in the wild type and single *icl* mutants but not in the *icl1 icl2* mutant. This demonstrated that *ICL2* encodes a specific 2-methylisocitrate lyase and that the *ICL1*-encoded isocitrate lyase exhibits a low but significant activity with 2-methylisocitrate. Subcellular fractionation studies and experiments with an ICL2-green fluorescent protein fusion demonstrated that the *ICL2*-encoded 2-methylisocitrate lyase is located in the mitochondrial matrix. Similar to that of *ICL1*, transcription of *ICL2* is subject to glucose catabolite repression. In glucose-limited cultures, growth with threonine as a nitrogen source resulted in a ca. threefold induction of *ICL2* mRNA levels and of 2-methylisocitrate lyase activity in cell extracts relative to cultures grown with ammonia as the nitrogen source. This is consistent with an involvement of the 2-methylcitrate cycle in threonine catabolism.

The complete sequencing of the *Saccharomyces cerevisiae* genome has yielded a large number of open reading frames with unknown function (11). Some of the newly discovered open reading frames exhibited a strong homology with known yeast genes and were demonstrated to encode hitherto-unknown isoenzymes. An example is the *PYK2* gene, which encodes a pyruvate kinase isoenzyme but can restore growth of *pyk1* null mutants on glucose only when overexpressed (3). In other cases, the biochemical function of the proteins (if any) encoded by the homologous open reading frames remains unknown.

An intriguing case is presented by the *ICL2* gene. This gene (YPR006c) exhibits a substantial sequence similarity with *ICL1* (13), the unique *S. cerevisiae* structural gene encoding isocitrate lyase (38% identity at the amino acid level). Isocitrate lyase is a key enzyme of the glyoxylate cycle. As this pathway is essential for growth on acetate and ethanol, *icl1* null mutants are unable to grow on ethanol or acetate (9, 28). *ICL2* is transcribed in ethanol-grown cultures of wild-type *S. cerevisiae*, and experiments with *ICL2-lacZ* fusions indicated that its transcriptional regulation is similar to that of *ICL1* (13). However, even the introduction of multiple copies of *ICL2* cannot complement the growth deficiency of *icl1* null mutants (13). This indicates that *ICL2* does not encode a functional isocitrate lyase. Since, furthermore, *icl2* null mutants have not been found to exhibit a discernible phenotype (13), the physiological function of *ICL2* has so far remained an enigma.

A reaction analogous to the conversion of isocitrate to glyoxylate and succinate occurs in the metabolism of propionyl-coenzyme A (CoA) via the 2-methylcitrate cycle (Fig. 1). This pathway was first discovered in alkane- and lipid-metabolizing yeasts (31, 32). An erroneous assumption about the pathway of propionyl-CoA metabolism in *S. cerevisiae* has caused some confusion. Based on the assumption that propionyl-CoA metabolism in this yeast involves *methyl*-malonyl-CoA as an intermediate, results from experiments with ^{13}C-labeled propionate were interpreted as proof of the occurrence of tight channeling of tricarboxylic acid (TCA) cycle intermediates (30). It was later shown that, instead, the methylcitrate cycle is the key pathway of propionate metabolism (23). The methylcitrate cycle is initiated by the synthesis of 2-methylcitrate from propionyl-CoA and oxaloacetate. 2-Methylcitrate is then converted into 2-methylisocitrate, which is subsequently split into pyruvate and succinate. The latter reaction is very similar to the conversion of isocitrate to succinate and glyoxylate, the reaction catalyzed by the *ICL1*-encoded isocitrate lyase.

The physiological function of the methylcitrate cycle in *S. cerevisiae* is not entirely clear. *S. cerevisiae* cannot grow on propionate as a sole carbon source, but in aerobic sugar-limited chemostat cultures, propionate can be cometabolized (23). It is also conceivable that this pathway may be involved in the

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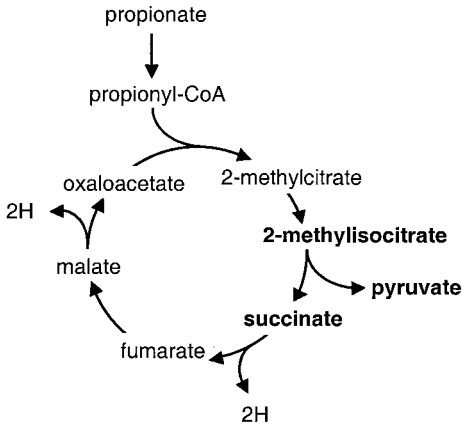

FIG. 1. 2-Methylcitrate cycle of propionyl-CoA metabolism (31, 32). The reaction catalyzed by 2-methylisocitrate lyase is shown in bold.

degradation of the carbon skeletons of certain amino acids. For example, oxidative decarboxylation of 2-ketobutyrate, an intermediate in threonine catabolism, yields propionyl-CoA (19).

In the present study, we tested the hypothesis that the *S. cerevisiae ICL2* gene encodes a specific 2-methylisocitrate lyase. Furthermore, the regulation of *ICL2* expression and the subcellular localization and physiological function of Icl2p were investigated.

MATERIALS AND METHODS

Yeast strains and maintenance. All *S. cerevisiae* strains used in this study are prototrophic members of the CEN.PK series and are described in Table 1. The strains were grown to stationary phase at 30°C in shake flask cultures on YPD medium (Difco yeast extract, 10 g per liter; Difco peptone, 20 g per liter; glucose, 20 g per liter). Subsequently, glycerol (20%, vol/vol) was added and 2-ml aliquots were stored at −70°C in sterile vials. Precultures were inoculated directly from these frozen stocks.

Construction of null mutants. Haploid *S. cerevisiae* null mutants were constructed by replacing the gene(s) of interest with a kanamycin resistance gene (the *kanMX* module) according to the PCR-based method of Wach et al. (39) as described previously (17). Mating type and replacement of genes by the *kanMX* module were verified by PCR as described previously (17). The sequences of the primers that were used for deletion (S1 and S2) and verification (A1, A4, K1, and K2) are listed in Table 2.

Mineral medium. The mineral medium used for batch and chemostat experiments contained the following per liter of demineralized water: $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; EDTA, 15 mg; $ZnSO_4 \cdot 7H_2O$, 4.5 mg; $CoCl_2 \cdot 6H_2O$, 0.3 mg; $MnCl_2 \cdot 2H_2O$, 0.84 mg; $CuSO_4 \cdot 5H_2O$, 0.3 mg; $CaCl_2 \cdot 2H_2O$, 4.5 mg; $FeSO_4 \cdot 7H_2O$, 3.0 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.4 mg; H_3BO_3, 1.0 mg; KI, 0.1 mg; and silicone antifoam (BDH), 0.15 ml. After autoclaving (120°C, 20 min), the medium was cooled to room temperature. Subsequently, filter-sterilized vitamins were added to the following final concentrations (per liter): biotin, 0.05 mg; calcium pantothenate, 1.0 mg; nicotinic acid, 1.0 mg; *myo*-inositol, 25.0 mg; thiamine-HCl, 1.0 mg; pyridoxol-HCl, 1.0 mg; and *para*-aminobenzoic acid, 0.2 mg. Glucose was sterilized separately for 20 min at 110°C, and ethanol was added without separate sterilization. Carbon substrates were added to a concentration of 250 mM carbon unless indicated otherwise. When L-threonine was used as a nitrogen source, the ammonium sulfate was replaced by an equimolar amount (based on nitrogen content) of this amino acid. To compensate for the reduced sulfate content of these media, 6.6 g of K_2SO_4 per liter was added as well. The mineral medium used for ammonium-limited chemostat cultivation contained a fivefold-reduced concentration of $(NH_4)_2SO_4$ (1.0 g per liter) and a fivefold-increased glucose concentration (37.5 g per liter, corresponding to 1.25 M carbon) and was supplemented with 5.3 g of K_2SO_4 per liter. Threonine-limited cultures were grown on the same medium with 1.8 g of L-threonine per liter instead of ammonium sulfate.

Shake flask cultivation. Shake flask cultures were grown at 30°C in 500-ml Erlenmeyer flasks on an orbital shaker (200 rpm). Precultures were prepared by inoculating 100 ml of YPD medium with a frozen-stock culture. After 48 h of incubation, a 1-ml sample was inoculated in a 500-ml Erlenmeyer flask containing 100 ml of mineral medium (pH 5.5). The composition of the mineral medium was as specified above, with the following modifications: ammonium sulfate was omitted and 30 mmol of L-aspartate and 62 mmol of ethanol per liter were added as carbon/nitrogen and carbon sources, respectively. After 24 h of incubation, a 2-ml sample was used to inoculate a second 100-ml culture on the same medium. This was again incubated for 24 h and subsequently used to prepare cell extracts for enzyme assays.

Chemostat cultivation. Chemostat cultivation was performed at 30°C in laboratory fermentors (Applikon, Schiedam, The Netherlands). The working volume of the cultures was kept at 1.0 liters by a peristaltic effluent pump coupled to an electrical level sensor; the stirrer speed was 800 rpm. The pH was kept constant at 5.0 by an ADI 1020 biocontroller via the automatic addition of 2 mol of KOH per liter. The gas flow through the cultures was maintained at 0.5 liters per min using a Brooks 5876 mass flow controller. The dissolved oxygen concentration was monitored with an autoclavable oxygen electrode (no. 34 100 3002; Ingold). Cultures were assumed to be in steady state when, after a change of growth conditions, at least five volume changes had passed and two subsequent samples taken at an interval of at least one volume change gave identical results for dry weight, CO_2 production rate, and O_2 consumption rate. Cultures were checked for purity using phase-contrast microscopy (×1,000 magnification) and by plating on YPD agar plates.

Gas analysis. The exhaust gas was cooled in a condenser (4°C) and dried in a Perma Pure dryer (PD-625-12P). O_2 consumption was determined with a Servomex 1100A oxygen analyzer (Taylor Servomex Co., Crowborough, United Kingdom). CO_2 production by the cultures was determined with a Beckman model 864 infrared detector. The CO_2 production and O_2 consumption rates were calculated according to the method of van Urk et al. (37).

Determination of culture dry weight. The dry weight of 10.0-ml culture samples was determined using 0.45-μm-pore-size nitrocellulose filters (Gelman Sciences) and a microwave oven (22). Duplicate samples varied by less than 1%.

Metabolite analysis. The concentration of glucose in reservoir media and supernatants was determined enzymatically using the UV method for D-glucose (no. 716 251; Boehringer Mannheim). The concentration of ethanol was determined enzymatically (38) using *Hansenula polymorpha* alcohol oxidase (kindly provided by Bird Engineering, Schiedam, The Netherlands). The concentration of other metabolites (organic acids and glycerol) were detected by high-performance liquid chromatography (Waters Alliance coupled to a dual-wavelength-absorbance and refractive-index detector) analysis on an Aminex HPX-87H column (Bio-Rad). The column was eluted at 60°C with 0.5 g of H_2SO_4 per liter at a flow rate of 0.6 ml · min^{-1}.

Preparation of cell extracts. Cells from shake flask cultures or chemostat cultures (ca. 80 mg [dry weight]) were harvested by centrifugation (4,000 × *g*, 10 min), washed once with 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM dithiothreitol (4°C), and resuspended in the same buffer. Cells were disrupted by sonication with 0.7-mm glass beads (0°C, 3 min; 30-s bursts with 30-s cooling intervals) using an MSE sonicator (150-W output, 7- to 8-μm peak-to-peak amplitude). Whole cells and debris were removed by centrifugation at 20,000 × *g* for 20 min at 4°C. The clear supernatant was used as the cell extract. Protein concentrations in cell extracts were determined by the Lowry method. Bovine serum albumin (fatty acid free; Sigma, St. Louis, Mo.) was used as a standard.

Enzyme assays. Enzyme activities were assayed at 30°C in a Hitachi model 100-60 spectrophotometer with freshly prepared cell extracts. The following

TABLE 1. *S. cerevisiae* strains used in this study[a]

Strain	Genotype
CEN.PK113-7D	*MATa URA3 HIS3 LEU2 TRP1 MAL2-8^c SUC2*
CEN.PK122	*MATa*/*MATα URA3*/*URA3 HIS3*/*HIS3 LEU2*/*LEU2 TRP1*/*TRP1 MAL2-8^c*/*MAL2-8^c SUC2*/*SUC2*
CEN.PK229-4D	*URA3 HIS3 LEU2 TRP1 MAL2-8^c SUC2 icl1Δ*
CEN.PK288-1C	*URA3 HIS3 LEU2 TRP1 MAL2-8^c SUC2 icl2Δ*
CEN.PK321-2D	*URA3 HIS3 LEU2 TRP1 MAL2-8^c SUC2 icl1Δ icl2Δ*
CEN.PK525-7D	*MATa URA3 HIS3 LEU2 TRP1 MAL2-8^c SUC2 ICL1 ICL2*::*yEGFP3-loxP-KanMX-loxP*

[a] All strains listed can be obtained from EUROSCARF (European *Saccharomyces cerevisiae* Archive for Functional Analysis) at http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/index.html.

enzymes were assayed according to previously published procedures: glucose-6-phosphate-dehydrogenase (EC 1.1.1.49 [22]), cytochrome-*c* oxidase (EC 1.9.3.1 [8]), citrate synthase and isocitrate lyase (EC 4.1.3.7 and EC 4.1.3.1 [6]), and catalase (EC 1.11.1.6 [35]). 2-Methylisocitrate lyase activity was assayed in a reaction mixture (1 ml) containing potassium phosphate buffer (pH 7.0) (100 μmol), phenylhydrazine (4 μmol), cysteine (2.5 μmol), $MgCl_2$ (2.5 μmol), and cell extract. The reaction was started by addition of 2-methylisocitrate (2 μmol). The molar extinction coefficient of pyruvate phenylhydrazone was experimentally determined to be 12 $mM^{-1} \cdot cm^{-1}$ (data not shown). Control experiments in which the rate of pyruvate production from 2-methylisocitrate was coupled to a NADH-linked lactate dehydrogenase gave identical specific activities (data not shown). In all enzyme assays, reaction rates were linearly proportional to the amount of cell extract added.

Northern experiments. Total RNA was extracted as previously described (26). Amplified PCR fragments of *ACT1*, *ICL1*, and *ICL2* were used as probes for Northern analysis. A ready-to-go DNA labeling kit (Pharmacia Biotech Europe) was used for DNA [α-^{32}P]dCTP radiolabeling. Total RNA (30 μg/lane) was separated on 1.2% agarose-formaldehyde gels, blotted to an Amersham Hybond-N membrane, and hybridized overnight at 42°C (27). After being washed, membranes were exposed to Kodak X-Omat MR film and incubated with an intensifying screen at −70°C.

Isolation of organelles. An organellar fraction was isolated from aerobic, glucose-limited chemostat cultures grown with threonine as the nitrogen source. The procedure, which involved differential centrifugation of cell homogenates obtained by controlled lysis of spheroplasts, has been described previously (17). To investigate the latency of enzymes in organellar fractions, enzyme activities were first measured in the presence of 0.65 mol of sorbitol per liter to osmotically stabilize the organelles. Subsequently, 0.1% Triton X-100 was added to disrupt the organelles and to measure intraorganellar enzyme activity. In independent control experiments, organelles were disrupted by sonication (150-W output, 30 s).

Sucrose density gradient centrifugation of organellar fractions. Cells were harvested by centrifugation at room temperature (1,600 × *g*, 5 min). For the generation of spheroplasts, cells were resuspended (0.1 g [wet weight] · ml^{-1}) in 0.1 M Tris buffer (pH 9.3) containing 10 mM dithiothreitol and incubated at 30°C for 10 min. After centrifugation (1,600 × *g*, 5 min), cells were washed once in a 50 mM potassium phosphate buffer (pH 7.2) containing 1.2 M sorbitol, resuspended in the same buffer containing 0.5 mg of Zymolyase 20T (ICN Biomedicals BV, Zoetermeer, The Netherlands) per ml, and incubated at 30°C for approximately 30 min. All subsequent steps were performed at 4°C. Spheroplasts were collected by centrifugation (2,800 × *g*, 7 min), washed in 5 mM morpholineethanesulfonic acid (MES) buffer (pH 5.5) containing 1.2 M sorbitol, and osmotically lysed by resuspension in 5 mM MES buffer (pH 5.5) containing 0.8 M sorbitol, 1 mM phenylmethylsulfonyl fluoride, and 2.5 mg of leupeptin per ml. After homogenization using a Potter-Elvehjem homogenizer, the suspension was adjusted to 1.2 M sorbitol by addition of 5 mM MES buffer (pH 5.5) containing 3.0 M sorbitol. The homogenate was subjected to two consecutive centrifugation runs (2,000 × *g*, 10 min, and 7,800 × *g*, 15 min). The resulting postnuclear supernatant was centrifuged (30,000 × *g*, 30 min) in order to obtain an organellar fraction, which was subsequently resuspended in 5 mM MES buffer (pH 5.5) containing 35% (wt/vol) sucrose. The organellar fraction was loaded onto a discontinuous sucrose density gradient consisting of 5 mM MES buffer (pH 5.5) containing 65% (wt/vol) sucrose (5 ml), 50% (wt/vol) sucrose (6 ml), 45% (wt/vol) sucrose (6 ml), 40% (wt/vol) sucrose (6 ml), sample, and 25% (wt/vol) sucrose (2-ml overlay). After centrifugation of the gradient in a vertical rotor (30,000 × *g*, 3 h), fractions of approximately 1.25 ml were taken from the gradient, starting at the highest sucrose concentration.

Experiments with an Icl-GFP fusion. To fuse the C terminus of Icl2p with the green fluorescent protein (GFP) gene, the yEGFP2-loxP-KanMX-loxP cassette from pUG30 (kindly provided by H. Hegemann, University of Düsseldorf, Düsseldorf, Germany) was amplified by PCR. The resulting construct, with ends homologous to the 3′ end of the *ICL2* gene, was then integrated at the *ICL2* locus of the wild-type strain, CEN.PK113-7D. Analysis of cells by fluorescence microscopy (using a Zeiss fluorescence microscope) and fixation and preparation for electron microscopy were performed as described previously (1, 40). Immunolabeling was performed on ultrathin sections of unicryl-embedded cells using specific antibodies against GFP and gold-conjugated goat anti-rabbit antibodies (40).

Sequence analysis. A search for a potential mitochondrial targeting sequence in Icl2p was performed with the PcGene software package (version 6.80; Intelligenetics, Mountain View, Calif.). This program is based on the method of Gavel and von Heijne (10).

RESULTS

***ICL2* encodes a specific 2-methylisocitrate lyase.** To investigate whether the *ICL2* gene encodes a specific 2-methylisocitrate lyase, activities of isocitrate lyase and 2-methylisocitrate lyase were determined in cell extracts of wild-type *S. cerevisiae* and isogenic *icl1*Δ, *icl2*Δ, and *icl1*Δ *icl2*Δ mutants. All strains were grown on ethanol in shake flask cultures to induce *ICL2* transcription (13). Aspartate was used as the sole nitrogen source and as an additional carbon source to circumvent the growth deficiency of *icl1* null mutants on ethanol-ammonia media (9, 28).

Cell extracts of the wild-type strain exhibited substantial activities of isocitrate lyase as well as 2-methylisocitrate lyase (Table 3). Inactivation of the *ICL1* gene completely abolished isocitrate lyase activity, confirming the earlier report that *ICL2* does not encode a functional isocitrate lyase (13). When, in addition to *ICL1*, *ICL2* also was deleted, the resulting strain completely lacked 2-methylisocitrate lyase activity as well as isocitrate lyase activity (Table 3). An *icl2* null mutant, which still exhibited high isocitrate lyase activity, also retained a residual activity of 2-methylisocitrate lyase (Table 3). These results demonstrate that the *S. cerevisiae ICL2* gene encodes a specific 2-methylisocitrate lyase and that the *ICL1*-encoded enzyme can utilize both isocitrate and 2-methylisocitrate as substrates.

***ICL2* expression is repressed by glucose and induced by threonine.** Regulation of *ICL2* expression was studied in steady-state chemostat cultures of the wild-type strain, *S. cer-*

TABLE 2. Oligonucleotides used for construction of disruption cassettes (S1 and S2) and as primers for analytical PCR of deletion mutants (A1-K1 and A4-K2)[a]

Gene	Oligonucleotide	Sequence
ICL1	S1	TGCCTATCCCCGTTGGAAATACGAAGAACGATTTTGCAGCAGCTGAAGCTTCGTACGC
ICL1	S2	TTCTTTACGCCATTTTCTTTGAATTGATCTTCTGTGACACGCATAGGCCACTAGTGGATCTG
ICL1	A1	GGAGAACTTCTAGCACGTTG
ICL1	A2	CGGCAGCATCTGCATCTAG
ICL1	A3	TCTGGTGCGGAGTACATCG
ICL1	A4	GTGTGTGTACATGTATGCGG
ICL2	S1	GGAACACTGAAGAAGCTGGTTTTGTCATCAGACAAGTCACCAGCTGAAGCTTCGTACGC
ICL2	S2	AAATTGCGTTTCGGTAAAGCTCTCACCAGATGTACTTAAAGCATAGGCCACTAGTGGATCTG
ICL2	A1	CAAGTAGTACCAGGAGTCACG
ICL2	A2	CTTGATTCCGAGAACACGAGG
ICL2	A3	CAGCTATGGTCTGGTGCAG
ICL2	A4	GAGAGTGTAGTCATGCAATCC
kanMX	K1	GGATGTATGGGCTAAATGTACG
kanMX	K2	GTTTCATTTGATGCTCGATGAG

[a] Construction of disruption cassettes and deletion of genes and analytical PCR were carried out as described previously (17). The sequences complementary to the multiple cloning site of pUG6 are underlined. The application of the different primers is explained in reference 17.

TABLE 3. Activity of isocitrate lyase and 2-methylisocitrate lyase in cell extracts of wild-type *S. cerevisiae* and of isogenic null mutants lacking *ICL1*, *ICL2*, or both[a]

Strain	Genotype	Activity (μmol · min^{-1} · mg of protein^{-1}) of:		me-ICL/ICL
		ICL	me-ICL	
CEN.PK113-7D	*ICL1 ICL2*	0.10	0.046	0.5
CEN.PK 229-4D	*icl1*Δ *ICL2*	<0.002	0.075	>37
CEN.PK 321-2D	*icl1*Δ *icl2*Δ	<0.002	<0.002	NA[b]
CEN.PK 288-1C	*ICL1 icl2*Δ	0.083	0.016	0.2

[a] All strains were grown in shake flask cultures on ethanol-aspartate medium. Data are from one set of shake flask cultures; activities in independent replicate experiments differed by less than 20%. ICL, isocitrate lyase; me-ICL, 2-methylisocitrate.
[b] NA, not applicable.

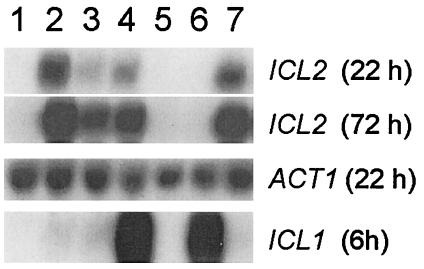

FIG. 2. Transcriptional regulation of *ICL1* and *ICL2* in chemostat cultures. Northern blots of mRNA isolated from chemostat cultures ($D = 0.10$ h^{-1}) grown under various nutrient limitation regimens were hybridized with *ICL1* and *ICL2* probes as well as with an *ACT1* reference probe. Numbers indicate growth conditions as follows: 1, aerobic, threonine-limited cultivation of the wild type with glucose as the carbon source; 2, aerobic, glucose-limited cultivation of the wild type with threonine as the nitrogen source; 3, aerobic, glucose-limited cultivation of the wild type with ammonia as the nitrogen source; 4, aerobic, ethanol-limited cultivation of the wild type with ammonia as the nitrogen source; 5, anaerobic, glucose-limited cultivation of the wild type with ammonia as the nitrogen source; 6, aerobic, ethanol-limited cultivation of the *icl2*Δ mutant with ammonia as the nitrogen source; 7, aerobic, glucose-limited cultivation of the wild type with threonine as the nitrogen source (independent duplicate of experiment under condition 2). Times in parentheses are autoradiography exposure times.

evisiae CEN.PK113-7D, grown at a fixed specific growth rate of 0.10 h^{-1}, by standard Northern analysis and by assaying the levels of isocitrate lyase and 2-methylisocitrate lyase in cell extracts.

Consistent with the results of shake flask cultures (Table 3), aerobic ethanol-limited chemostat cultures exhibited a substantial activity of isocitrate lyase as well as of 2-methylisocitrate lyase (Table 4). Under this cultivation condition, both *ICL* genes yielded a clearly detectable signal on Northern blots (Fig. 2). Aerobic cultivation on glucose as the growth-limiting substrate yielded a circa 15-fold-lower isocitrate lyase activity than growth on ethanol (Table 4) and on Northern blots yielded only a faint signal of the *ICL1* transcript (Fig. 2). The activity of 2-methylisocitrate lyase in glucose-limited chemostat cultures was circa threefold lower than that in the ethanol-limited cultures, consistent with a similar difference in *ICL2* transcript levels (Table 4 and Fig. 2). Both enzyme activities, as well as the *ICL1* and *ICL2* transcripts, were undetectable in cultures grown on glucose with ammonium sulfate as the growth-limiting nutrient (Table 4 and Fig. 2). Apparently, the high residual glucose concentrations in such cultures (28.7 g per liter) (data not shown) led to glucose catabolite repression of both *ICL* genes. Neither enzyme activity nor transcripts were detected in anaerobic glucose-limited cultures.

Propionyl-CoA, the substrate of the 2-methylcitrate cycle in which 2-methylisocitrate lyase participates, may be formed as an intermediate of threonine catabolism. Indeed, glucose-limited cultivation with threonine instead of ammonia as the nitrogen source led to a circa threefold increase of activity of 2-methylisocitrate lyase in cell extracts and of the *ICL2* transcript level (Table 4 and Fig. 2). No inducing effect of threonine was observed for expression of *ICL1* (Table 4 and Fig. 2). No *ICL2* transcript or 2-methylisocitrate lyase activity was detectable during threonine-limited growth on glucose (Table 4 and Fig. 2). As the residual glucose concentration in these cultures, was 13.7 g per liter, this indicates that induction of *ICL2* expression by threonine was overruled by glucose catabolite repression.

2-Methylisocitrate lyase is a mitochondrial matrix protein. Initial subcellular fractionation experiments were performed by differential centrifugation of cell homogenates prepared from glucose-limited chemostat cultures grown with threonine as the nitrogen source. As discussed above, the 2-methylisocitrate lyase in such cultures was exclusively encoded by the *ICL2* gene (Table 4). In four independent experiments, 60 to 80% of the 2-methylisocitrate lyase activity in the cell homogenates was recovered in the particulate fraction. A similar incomplete recovery in the particulate fraction was found for citrate synthase, which in *S. cerevisiae* is known to be confined to mitochondria and/or microbody matrices. For both enzymes, 20 to 40% of the total activity was recovered in the soluble fraction of the homogenates. As the mitochondrial inner membrane protein cytochrome-*c* oxidase in the same experiments was completely (>90%) recovered in the particulate fraction, the most probable explanation for the incomplete recovery of citrate synthase is that damage to some or-

TABLE 4. Activities of isocitrate lyase and 2-methylisocitrate lyase in cell extracts of *S. cerevisiae* CEN.PK113-7D in steady-state chemostat cultures[a]

Carbon source	Nitrogen source	Activity (μmol · min^{-1} · mg of protein^{-1}) of:		me-ICL/ICL
		ICL	me-ICL	
Ethanol	Ammonia	0.15 ± 0.02	0.09 ± 0.00	0.63
Glucose	Ammonia	0.01 ± 0.00	0.03 ± 0.00	3
Glucose	**Ammonia**	<0.002	<0.002	NA[b]
Glucose (anaerobic)	Ammonia	<0.002	<0.002	NA
Glucose	Threonine	<0.002	0.10 ± 0.01	>50
Glucose	**Threonine**	<0.002	<0.002	NA

[a] The growth-limiting substrates are printed in bold. Unless otherwise indicated, cultures were aerobic (dissolved oxygen concentration above 60% of air saturation). Data are presented as the ± the standard deviations of two independent chemostat cultivations. $D = 0.10$ h^{-1}.
[b] NA, not applicable.

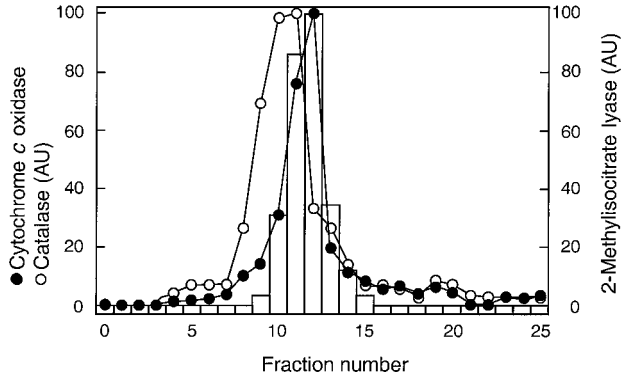

FIG. 3. Sedimentation patterns of 2-methylisocitrate lyase in sucrose density gradients. An organellar fraction isolated from a glucose-limited, aerobic chemostat culture ($D = 0.10\ h^{-1}$) grown with threonine as the nitrogen source was subjected to sucrose density gradient centrifugation (see Materials and Methods). Fraction 1 corresponds to the bottom fraction of the gradient. Cytochrome-*c* oxidase and catalase were used as mitochondrial and microbody marker enzymes, respectively. Enzymes are plotted as a percentage of the activity in the peak fraction. A replicate experiment yielded the same results (data not shown). AU, arbitrary units.

ganelles during the fractionation experiments led to some leakage of matrix enzymes. The cytosolic marker enzyme glucose-6-phosphate dehydrogenase was exclusively recovered in the cytosol.

2-Methylisocitrate lyase activity in organellar fractions exhibited latency. When organelles were osmotically stabilized during the enzyme assays, only a very low activity was measured. This activity increased 18- to 20-fold when organelles were disrupted prior to the enzyme assays by 30 s of sonication or by addition of Triton X-100. This indicated that 2-methylisocitrate lyase is located in an organellar matrix.

To investigate in which organelle 2-methylisocitrate lyase is located, the particulate fraction of a cell homogenate was subjected to sucrose density gradient centrifugation. In the density gradient centrifugation, catalase and cytochrome-*c* oxidase were used as marker proteins for the microbody and mitochondrial fractions, respectively (7, 35). These marker proteins showed clearly different sedimentation patterns, with catalase sedimenting at higher average sucrose concentrations than cytochrome-*c* oxidase (Fig. 3). The sedimentation pattern of 2-methylisocitrate lyase perfectly matched that of the mitochondrial marker enzyme cytochrome-*c* oxidase, indicating that the *ICL2* gene product is a mitochondrial matrix enzyme. To verify this conclusion, the C-terminal end of Icl2p was fused to GFP. Fluorescence microscopy of intact cells as well as immunogold labeling of thin sections confirmed that the fusion protein was targeted to the mitochondrial matrix (Fig. 4). Indeed, analysis of the predicted protein sequence of Icl2p (10) revealed a potential N-terminal mitochondrial transit peptide from positions 1 to 32.

DISCUSSION

Since its discovery in alkane- and lipid-metabolizing yeasts (31, 32), the methylcitrate cycle has also been shown to be the key pathway of propionyl-CoA metabolism in the *Enterobacteriaceae Escherichia coli* and *Salmonella enterica* serovar Typhimurium (14, 16, 33). Biochemical evidence indicates the existence in lipid- and alkane-metabolizing yeasts of dedicated methylcitrate cycle enzymes different from their counterparts in the TCA and glyoxylate cycles (34). In *Enterobacteriaceae*, propionate metabolism operons harbor genes encoding key enzymes of the pathway (propionyl-CoA synthetase, 2-methylcitrate synthase, an aconitase, and a 2-methylisocitrate lyase) (14, 33).

The identification of a specific 2-methylisocitrate lyase in *S. cerevisiae* was surprising, as previous studies seemed to indicate that, in this yeast, the reactions of propionate metabolism can be catalyzed by enzymes involved in the analogous reactions of acetyl-CoA metabolism. In *S. cerevisiae*, activation of propionate to propionyl-CoA can be catalyzed by the *ACS1*-encoded isoenzyme of acetyl-CoA synthetase (36), and propionyl-CoA is a substrate for the acetylcarnitine transferase shuttle (21). Likely candidate genes for the 2-methylcitrate synthase-encoding gene are *CIT1*, *CIT2*, and *CIT3*, which all encode active citrate synthase isoenzymes (15). In glucose-limited chemostat cultures fed with increasing concentrations of propionate as a cosubstrate, induction of 2-methylcitrate synthase activity was paralleled by an increase in citrate synthase activity (23). This is consistent with the involvement of one or more of the citrate synthase isoenzymes in the 2-methylcitrate synthase reaction. Candidate structural genes for the aconitase-like enzyme of the 2-methylcitrate cycle include *ACO1* (the structural gene for aconitase), YJL200c (the predicted polypeptide product of which exhibits 55% amino acid identity with *ACO1* [25]), and *LYS4* (the structural gene for homocitrate dehydratase).

McFadden et al. (18) reported that the activities of purified isocitrate lyase from *S. cerevisiae* with 2-methylisocitrate and isocitrate exhibited a 1-to-5 ratio. This is in good agreement with the ratio of 2-methylisocitrate lyase and isocitrate lyase activities found in cell extracts of an *icl2* null mutant (Table 3). Even though the *ICL1*-encoded isocitrate lyase can catalyze

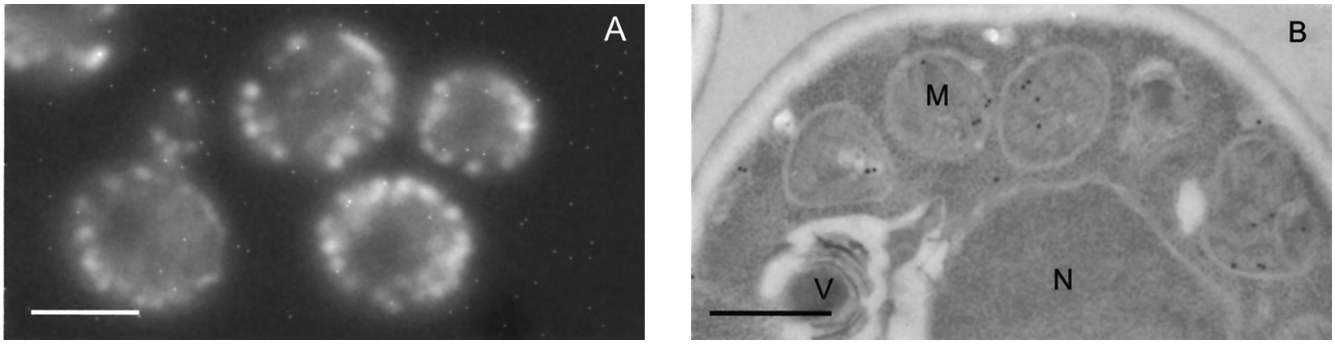

FIG. 4. Localization of an Icl1-GFP fusion. *S. cerevisiae* CEN.PK525-7D (*ICL2*::*yEGFP3*) was pregrown on ethanol-aspartate medium. (A) Fluorescence microscopy. Bar, 2 μm. (B) Electron micrograph of a thin section, labeled with anti-GFP antiserum and goat anti-rabbit antibodies linked to gold particles. Abbreviations: M, mitochondria; N, nucleus; V, vacuole. Specific labeling is exclusively located on the mitochondrial matrix. Bar, 0.5 μm.

the conversion of 2-methylisocitrate to succinate and pyruvate, our data indicate that *S. cerevisiae* contains a specific 2-methylisocitrate lyase encoded by *ICL2*. A factor that may have contributed to the evolution of a highly specific mitochondrial 2-methylisocitrate lyase in *S. cerevisiae* is that any isocitrate lyase activity of Icl2p could lead to intramitochondrial accumulation of glyoxylate. Glyoxylate, the product of the isocitrate lyase reaction, is an inhibitor of yeast citrate synthase (12). The presence of a mitochondrial 2-methylisocitrate lyase also prevents inhibition of TCA cycle enzymes by the C_7 intermediates 2-methylcitrate and 2-methylisocitrate, an effect described for human and bovine TCA cycle enzymes (2, 5).

In contrast to the other microorganisms in which the methylcitrate cycle has been studied, *S. cerevisiae* cannot grow on propionate as the sole carbon source (23). This is peculiar, since the 2-methylcitrate cycle oxidizes propionyl-CoA to pyruvate (Fig. 1), which does support growth of *S. cerevisiae*. Growth on propionate requires that part of the pyruvate formed in the 2-methylcitrate cycle be carboxylated to oxaloacetate, a key precursor in biosynthesis. Since, in contrast to the mitochondrial localization of Icl2p, both isoenzymes of pyruvate carboxylase are exclusively cytosolic (24), this requires export of pyruvate from the mitochondria. Biochemical evidence demonstrates that mitochondrial pyruvate import in *S. cerevisiae* is a carrier-mediated process (20), but it is unknown whether pyruvate transport is reversible in vivo. If pyruvate transport across the mitochondrial inner membrane is unidirectional (i.e., catalyzing only the import of pyruvate from the cytosol), the mitochondrial localization of Icl2p might preclude growth on propionate as the sole carbon source.

The induction of *ICL2* expression in glucose-limited cultures (Fig. 2 and Table 4) grown with L-threonine as the nitrogen source suggests that the main physiological role of the methylcitrate cycle in *S. cerevisiae* is the metabolism of endogenous propionyl-CoA. Conversion of threonine into propionyl-CoA is initiated by its deamination to 2-oxobutyrate, catalyzed by threonine dehydratase (encoded by the *CHA1* gene [4]). The subsequent oxidative decarboxylation of 2-oxobutyrate to propionyl-CoA can be catalyzed by the yeast mitochondrial branched-chain 2-oxo-acid dehydrogenase complex (29). From a physiological perspective, it is not illogical that *ICL2* is subject to glucose catabolite repression: efficient recovery of the carbon skeletons of amino acids is unlikely to be a major advantage under conditions of glucose excess. Whether additional potential sources of propionyl-CoA other than threonine catabolism, such as the catabolism of isoleucine or β-oxidation of odd-chain fatty acids (19), also feed the methylcitrate cycle of *S. cerevisiae* remains to be investigated.

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